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The Effects of Sperm Concentration, Sperm:Egg Ratio, and Gamete Age on Fertilization Success in Crown-of-Thorns Starfish (*Acanthaster planci*) in the Laboratory

J. A. H. BENZIE* AND P. DIXON

Australian Institute of Marine Science, PMB No. 3, Townsville MC, Queensland 4810, Australia

Abstract. Laboratory experiments varying gamete concentrations and gamete age demonstrated significant reductions in fertilization success of the starfish *Acanthaster planci* (L.) with decreasing sperm concentration and increasing age of both eggs and sperm. The effect of aging in sperm was faster than that of eggs, and the speed of sperm aging increased with increasing dilution of sperm. Fertilization success was high over a wide range of sperm: egg ratios but declined rapidly at ratios less than 50, particularly at low sperm concentrations. *A. planci* gametes aged more slowly, and the loss of fertilizing capacity of sperm with dilution (the respiratory dilution effect) was far less, than in sea urchins. These characteristics provide a mechanism for enhanced fertilization success at given sperm concentrations and at greater distances and times from the point of gamete release, and may explain the higher fertilization rates achieved over longer distances in the wild by *A. planci* relative to sea urchins. Gametes would remain competent for longer periods at more dilute concentrations and so better achieve long-distance fertilization. Gametes obtained at the end of the breeding season were qualitatively different from those obtained early in the breeding season and showed reduced fertilization success for a given combination of variables, and different fertilization dynamics.

Introduction

Most work on sea urchins and starfish has concentrated on the biochemical and cellular mechanisms underlying gamete maturation and fertilization and early larval development, using a few model species (Ishikawa, 1975; Chia and Bickell, 1983; Kanatani and Nagahama, 1983; Meijer and Guerrier, 1984). Reports on the effects of gamete age and concentration on fertilization success are mostly limited to laboratory studies and rarely address the process in an ecological context (Tyler and Tyler, 1966; Pennington, 1985; Levitan *et al.*, 1991).

Fertilization is affected by the age of the gametes (the time since they were first released into the seawater), the concentration of sperm, and the number of sperm relative to the number of eggs (Lillie, 1915, 1919; Cohn, 1918; Gray, 1928). Additional information on the effect of gamete concentrations on fertilization success has been obtained incidentally from studies using sea urchin sperm as a toxicological assay (Greenwood and Bennett, 1981; Kobayashi, 1984; Dinnel *et al.*, 1987). Pennington (1985) was the first to study fertilization success in the field. He confirmed the loss of fertilizing capacity of sperm of sea urchins with dilution and with increasing gamete age in the laboratory and inferred that the effects of sperm dilution were largely responsible for the marked reductions in fertilization success he observed in field experiments as distance between eggs and spawning males increased. Pennington's theory has been supported by further studies on sea urchins (Levitan, 1991; Levitan *et al.*, 1992), and similar explanations have been advanced to explain rapid reductions in fertilization success with distance in hydroids (Yund, 1990) and ascidians (Grosberg, 1991).

Despite the importance of gamete age and sperm concentration in determining fertilization success between individuals spatially separated to different extents, only one study has attempted to quantify experimentally the conjoint effects of several variables on fertilization success

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* Author to whom correspondence should be addressed.

(Levitan *et al.*, 1991). These authors demonstrated that the theoretical model developed by Vogel *et al.* (1982) accounted for 91% of the variation they observed in fertilization success.

The aim of the present work was to obtain conjoint information on the effects of sperm concentration, sperm: egg ratio, and gamete age on fertilization success in the crown-of-thorns starfish, *Acanthaster planci* (L.). Fertilization dynamics in this species is of particular interest because its large increases in population size (outbreaks), which have caused considerable damage to coral reefs throughout the world (Moran, 1986), could result from fluctuations in fertilization success. Despite intensive research in recent times, many aspects of *A. planci* biology—including fertilization dynamics—are still unknown (Lassig and Kelleher, 1991).

Although *A. planci* has been spawned and reared in captivity, fertilization success was not reported (Yamaguchi, 1974; Lucas, 1982). Information on spawning and fertilization rates in the field has been reported (Babcock and Mundy, 1992), but no studies have examined the influence of gamete characteristics or gamete concentrations on fertilization success. Comprehensive information on the conjoint effects of gamete concentrations and gamete age on fertilization success in *A. planci* is not only specifically relevant to the occurrence of outbreaks in the crown-of-thorns starfish but also adds to the limited information available on the effect of these variables on external fertilization in marine organisms.

Materials and Methods

Starfish were collected from Davies Reef (18 °50′ S, 147 °39′ E) in the Great Barrier Reef on the east coast of Australia from November to February in 1987, 1988, and 1989, in each of the breeding seasons for *A. planci*. Animals were sexed, and their breeding condition assessed, by making a small cut in the proximal part of one arm of the starfish and examining the gonad. Individuals with well-developed gonads were then kept separately in clear acrylic tanks with fresh-flowing seawater until they were used in the experiments. The seawater used in this treatment and in all experiments was approximately 28°C and pH 8.3. The usual range in summer values for the Great Barrier Reef inshore waters is 28–33°C and pH 8.2–8.6.

Gametes were obtained from portions of gonad approximately 5 cm^3 in volume that were removed from individual starfish through a 2-cm cut in the dorsal margin of one side of the proximal part of an arm. The tissue was placed in 100 ml of filtered (0.4 μm) seawater (FSW) containing 10^{-6} *M* 1-methyladenine to induce gamete release. Gonad tissue from female starfish was collected 1 h before gonad tissue from males because females responded more slowly than males to hormonal induction (approximately 45 min for females compared with less than 30 min for males) and eggs had to be counted into beakers before sperm were added. Only gametes extruded during the first 10–15 min in response to the hormonal treatment were used in the experiments.

Eggs were pipetted into a shallow crystallizing dish with clean FSW. After eggs had matured (as evidenced by germinal vesicle breakdown), they were counted into separate beakers for the various experimental treatments. For treatments requiring small numbers of eggs (80 or fewer), eggs were counted out individually from pasteur pipettes. For treatments requiring 100 eggs or more, a monolayer of eggs was settled on the base of the dish over a grid in which each square contained 100 eggs. With the aid of a dissecting microscope, eggs from the appropriate number of squares were sucked into a pasteur pipette and added to the test container.

The sperm solution was separated from male gonad tissue by decanting the solution into another beaker. This original 100 ml of concentrated solution usually contained 12–40 $\times$ 10^6 sperm ml^{-1}. Because 2.5 ml of tissue remained after sperm release, these figures suggest that sperm were stored and extruded at densities of 2 $\times$ 10^8–10^9 sperm ml^{-1}. Given that the extrusion occurred over 20 min, the average rate of sperm release from excised gonad was approximately 10^5–10^6 sperm s^{-1} One milliliter of well-mixed solution was removed and diluted 500 times in FSW to which formalin had been added to fix the sperm before counting. Two drops of solution were placed on a hemacytometer and the number of sperm counted. These data were used to calculate the appropriate aliquot of the original concentrated sperm solution to be diluted with FSW to provide 500 ml of a stock solution containing 2 $\times$ 10^6 sperm ml^{-1}. This stock solution was used as the base for serial dilutions to provide sperm of varying concentrations.

Preliminary tests had shown that sperm maintained at a concentration of 2 $\times$ 10^6 sperm ml^{-1} retained full fertilizing capacity for at least 24 h, but the longest period over which the solution was used in any experiment was less than 8 h. Rates of fertilization at a variety of dilutions from such stock solutions held for 8 h were the same as those from the same dilutions made immediately after the stock solution was created. In other words, stock solutions of 2 $\times$ 10^6 sperm ml^{-1} did not appear to "age" over 24 h. Timing was therefore carried out from the time of dilution from the stock solution held at 2 $\times$ 10^6 sperm ml^{-1}. This approach provided a clear and reproducible start to experiments with "non-aged" sperm.

There was no correlation between initial concentrations at which sperm had been spawned and the relative speed of aging of the sperm in subsequent experiments; the existence of a correlation might suggest that the initial spawning into seawater had irreversibly aged the sperm.

It should be noted that when sperm were released, they accumulated in a thick sludge at the base of the beaker and were therefore accumulating in very high concentrations. The values of 12–40 $\times$ 10^6 sperm ml^{-1} were observed after thorough mixing of the seawater in the beaker at the end of the spawning period.

Serial dilutions were used to obtain stock solutions of each sperm concentration immediately prior to first adding sperm to the eggs. To obtain the desired final concentration of sperm, 1 ml of sperm stock solution was added to every 19 ml of FSW containing the eggs. Stock sperm concentrations derived from 1, 1:10, 1:100, 1:1000, and 1:2000 dilutions of the 2 $\times$ 10^6 sperm ml^{-1} solution provided respective final concentrations of sperm of 10^5, 10^4, 10^3, 10^2, and 50 sperm ml^{-1} in the FSW containing the eggs. Most of the treatments were carried out in 50-ml glass beakers with 19 ml of FSW. However, where a specified sperm:egg ratio would have resulted in less than 10 eggs in 19 ml of FSW, volumes up to 1 liter were used to provide treatments having at least 10 eggs.

Preliminary experiments had demonstrated that no fertilization occurred if sperm concentrations were 10 sperm ml^{-1} or less, and that the proportion of eggs fertilized in any treatment did not increase after 45 min. In all experiments, therefore, only sperm concentrations greater than 10 sperm ml^{-1} were used, and the fertilized eggs were counted 45 min after sperm was first added to the eggs. In treatments using small containers, all eggs were counted and the number of fertilized eggs (those with a raised fertilization membrane) was noted, with the aid of a dissecting microscope. In treatments with large volumes of seawater, the eggs were collected using a pasteur pipette and placed in a small volume of seawater where those with a raised fertilization membrane were counted.

Preliminary experiments also demonstrated marked differences in performance by different individuals of one sex when mated with a given individual of the opposite sex and showed that the ranking of performance varied with the individual of the opposite sex used. Because experimental results could be strongly influenced by using one individual in several matings, subsequent experiments used an individual only once.

Variation in fertilization success at different times and in different years

The logistic difficulties in undertaking the large-scale combinations of sperm concentration, sperm:egg ratio, and differently aged gametes meant that data were collected from experiments conducted at different times in the breeding season and from more than one breeding season. To assess the effects of these variations on fertilization success, data from each time period were obtained on fertilization success at 0, 1, and 2 h after gamete release at sperm:egg ratios of 5000 and sperm concentrations of 10^4 sperm ml^{-1}.

Sperm concentration and sperm:egg ratio experiments

Experiments testing the effects of sperm concentration and sperm:egg ratio used final sperm concentrations of 50, 10^2, 10^3, 10^4, and 10^5 sperm ml^{-1} and sperm:egg ratios of 100, 500, 1000, 2500, and 5000 in every combination, giving a total of 25 treatments. Testing a number of the low sperm:egg ratios at sperm concentrations less than 50 sperm ml^{-1} was impracticable: it would have required several replicates of 10 l or more with only 10 eggs that would have been difficult to relocate and score.

Eggs were collected, and sperm solutions were diluted, immediately before the experiment and were used throughout the experiment, so that both gametes were aging over the course of the experiment. Sperm from the diluted stock concentrations was added to sets of eggs at 0 min, 15 min, 45 min, 1 h 45 min, and every succeeding hour until 6 h 45 min from the first insemination. Two replicates of each test combination were performed per mating, and a total of 10 different matings were used.

Gamete aging experiments

The number of sperm dilutions and sperm:egg ratios was reduced in experiments testing the independent effects of aging in either eggs or sperm because four combinations of "aging" and "fresh" gametes had to be tested. At each time interval, aging sperm was added to aging eggs as in the sperm concentration and sperm:egg ratio experiment. Aging sperm was also used to fertilize a newly collected set of fresh eggs from ovary tissue induced to spawn 45 min prior to that insemination. Freshly diluted stock solutions of fresh sperm were prepared from the 2 $\times$ 10^6 sperm ml^{-1} stock and added to both aging and fresh eggs. The sperm concentrations used were 10^2 and 10^4 sperm ml^{-1} and the sperm:egg ratios were 100, 1000, and 5000; with four combinations of gametes, this gave a total of 24 treatments. Sperm was added to sets of eggs at 0 min, 45 min, 1 h 45 min, and every succeeding hour until 6 h 45 min from the first insemination. Two replicates of each test combination were performed per mating, and a total of six different matings were used.

Statistical analysis

All results were expressed as percent fertilization and were arcsin transformed prior to analysis of variance and stepwise multiple regression using BMDP (Dixon *et al.*, 1983) and SAS (SAS Inc., 1985). Multidimensional plots were produced using graphic programs in SAS.

Table I

Analysis of variance testing for differences between early and late breeding season (time in breeding season), breeding seasons (year), and time since gamete release (gamete age), on fertilization success of Acanthaster planci

Source of variance	Degrees of freedom	Mean square	$F_{0,10}$
Time in breeding season	1	4.61	44.9***
Gamete age	2	0.86	8.4***
Year	1	0.12	1.2^{NS}
Time in breeding season × gamete age	2	0.17	1.7^{NS}
Time in breeding season × year	1	0.67	6.5*
Gamete age × year	2	0.17	1.7^{NS}
Time in breeding season × gamete age × year	2	0.08	0.8^{NS}
Error	49	0.10	

Data were from breeding seasons 2 and 3, fertilization at 0, 1, and 2 h from gamete release, at sperm: egg concentration of 5000 and final sperm concentration of 10^4 sperm ml^{-1} (see Table II for means).

* $P < 0.05$; *** $P < 0.001$; ^{NS} not significant.

Results

Variation in fertilization success at different times in the breeding season and in different years

Analysis of variance demonstrated that time after the gametes were released (gamete age) and period in the breeding season when the mating was made (time in breeding season) had highly significant effects on fertilization success, but that year had no independent effect (Table I). These results reflected the consistent drop in fertilization success with gamete age, whether early or late in the breeding season, and in every year (Table II). Similarly, fertilization rates were consistently lower at the end of the breeding season than early in the breeding season in every year. The significant interaction of time in breeding season with year reflected the fact that the difference in fertilization rates early and late in the breeding season was not the same from year to year.

Sperm concentration and sperm:egg ratio experiments

Data from two breeding seasons were pooled to increase the number of matings included in these tests but, because time at which mating took place within the breeding season had been shown to have a significant influence on fertilization rates, time in breeding season was included as a factor in the analysis. Sperm concentration, sperm: egg ratio, time after gamete release (gamete age), and time in breeding season had highly significant independent effects on percent fertilization (Table III). The large number of significant interaction terms demanded that care be taken in the interpretation of the main effects and indicated that, although the direction of change was the same for various combinations of factors, the degree of change was not. The principal pattern observed in the interaction terms for the analysis of the total data set was the lack of significance, or the lower significance, of interactions including sperm:egg ratio.

Stepwise multiple regression showed that gamete age, sperm concentration, and time in the breeding season all had significant effects, but that sperm:egg ratio had only a slight effect. R^2 values indicated that these variables cumulatively explained 45.2, 56.3, 63.7, and 64.2% of the variation, respectively.

Interpretation of the analysis of variance was assisted by reference to additional analyses in which the data set was first restricted to times less than 3.75 h and then to times less than 3.75 h and sperm concentrations greater than 10^3 sperm ml^{-1}. The restricted analyses were carried out because a large number of the treatments had zero

Table II

Mean percent fertilization rates (± standard errors) early and late in the Acanthaster planci *breeding season for gametes fertilized at 0, 1, and 2 h after gamete release*

Time after gamete release (hours)	Breeding season 1		Breeding season 2		Breeding season 3	
	Early (Nov–Dec 1986)	Late (Jan 1987)	Early (Nov–Dec 1987)	Late (Jan 1988)	Early (Nov–Dec 1988)	Late (Jan 1989)
0	–	94 ± 2 (3)	99 ± 1 (7)	79 ± 11 (5)	97 ± 2 (8)	67 ± 23 (2)
1	—	79 ± 16 (3)	92 ± 3 (7)	86 ± 8 (5)	94 ± 3 (8)	42 ± 39 (2)
2	—	54 ± 19 (3)	74 ± 24 (2)	31 ± 3 (5)	92 ± 7 (8)	19 ± 9 (2)

The sperm:egg ratio was 5000 and the final sperm concentration 10^4 sperm ml^{-1} in all tests. The sample size of independent matings is given in parentheses.

Table III

Analysis of variance testing the effects of time since initial gamete release (gamete age), sperm:egg ratio, sperm concentration, and time within breeding season (time in breeding season) on fertilization success in Acanthaster planci

Source of variance	Total data set			Time < 3.75 h			Time < 3.75 h, sperm concentration > 1000		
	Degrees of freedom (DF)	Mean square (MS)	$F_{0.07}$	DF	MS	$F_{0.11}$	DF	MS	$F_{0.14}$
Gamete age (G)	8	48.98	723.6***	4	27.97	260.8***	4	7.15	52.7***
Sperm:egg ratio (R)	4	1.61	23.7***	4	2.48	23.1***	4	0.24	1.8^{NS}
Sperm concentration (C)	4	22.00	325.0***	4	25.13	254.3***	1	1.58	11.7***
Time in breeding season (T)	1	60.43	892.7***	1	77.84	725.8***	1	17.37	128.2***
G × R	32	0.19	2.9***	16	0.16	1.5^{NS}	16	0.03	0.2^{NS}
G × C	32	1.16	17.1***	16	0.77	7.2***	4	0.31	2.3^{NS}
R × C	16	0.13	1.9*	16	0.27	2.5***	4	0.05	0.4^{NS}
G × T	8	3.46	51.1***	4	1.58	14.7***	4	0.26	1.9^{NS}
R × T	4	0.16	2.3^{NS}	4	0.22	2.0^{NS}	4	0.02	0.1^{NS}
C × T	4	0.43	6.3***	4	1.46	13.6***	1	1.61	11.9***
G × R × C	128	0.06	0.8^{NS}	64	0.07	0.6^{NS}	16	0.04	0.3^{NS}
G × R × T	32	0.06	0.9^{NS}	16	0.10	1.0^{NS}	16	0.04	0.3^{NS}
G × C × T	32	0.63	9.3***	16	0.78	7.3***	4	0.65	4.8***
R × C × T	16	0.09	1.4^{NS}	16	0.16	1.5^{NS}	4	0.04	0.3^{NS}
G × R × C × T	128	0.04	0.6^{NS}	64	0.06	0.5^{NS}	16	0.03	0.2^{NS}
Error	1800	0.07		1000	0.11		400	0.14	

* $P < 0.05$; *** $P < 0.001$; ^{NS} not significant.

fertilization values for many of the longer times after gamete release. It was thought that these values might depress the value of the error term and result in many significant interaction terms. Similarly, many of the lower sperm concentrations provided many zero fertilization records.

Analysis of variance for time restricted to less than 3.75 h showed the same pattern of effects as the complete analysis except for the loss of a significant interaction of gamete age and sperm:egg ratio. In the analysis additionally restricted to higher sperm concentrations, the significance of the main effects of sperm:egg ratio was lost.

Significant interaction terms were observed only for a two-way interaction of sperm concentration and time in breeding season and a three-way interaction of gamete age, sperm concentration, and time in breeding season.

Graphs illustrating fertilization at different times after gamete release for matings made early and late in the breeding season showed a consistent reduction in fertilization rates at all combinations of sperm concentration and sperm:egg ratios in the late breeding season (Fig. 1). Similarly, the higher the sperm concentration, the higher the degree and the greater the persistence of fertilization success. Fertilization rate decreased as gamete age increased, in every combination of factors. The pattern in each graph was similar for every sperm:egg ratio at sperm concentrations greater than 10^3 sperm ml^{-1}, indicating the reason for the lack of significant effects of sperm:egg ratio in the analysis restricted to high sperm concentrations and short times since gamete release. Fertilization rates were lower at lower sperm:egg ratios for given sperm concentrations, particularly for lower sperm concentrations and older gametes. This identified the source of the main effect of sperm:egg ratio in the analysis of the complete data set, the significant sperm:egg ratio × sperm concentration interactions in the analysis restricted to shorter times since gamete release, and an additional sperm:egg ratio × gamete age interaction in the full analysis. Similarly, the strong two- and three-way interactions of gamete age, sperm concentration, and time in breeding season reflected the progressively lower fertilization rates with increased gamete age and decreased sperm concentrations, as well as the exacerbating effect that mating late in the breeding season had on the depressing influence of these factors on fertilization.

The graphs in Figure 1 represent sections taken parallel to the time axis through the fertilization rate response surfaces plotted in Figure 2 as a function of sperm concentration and time since gamete release for different sperm:egg ratios. The surfaces for sperm:egg ratios of 5000 and 500 in the early breeding season were similar, whereas those from the late breeding season showed a reduction in fertilization rates at sperm:egg ratios of 500 relative to those at sperm:egg ratios of 5000 at short time intervals from gamete release. The surfaces from the late breeding season also showed a marked reduction in fertilization relative to those from the early breeding season, as well as the absence of a plateau of high fertilization at short

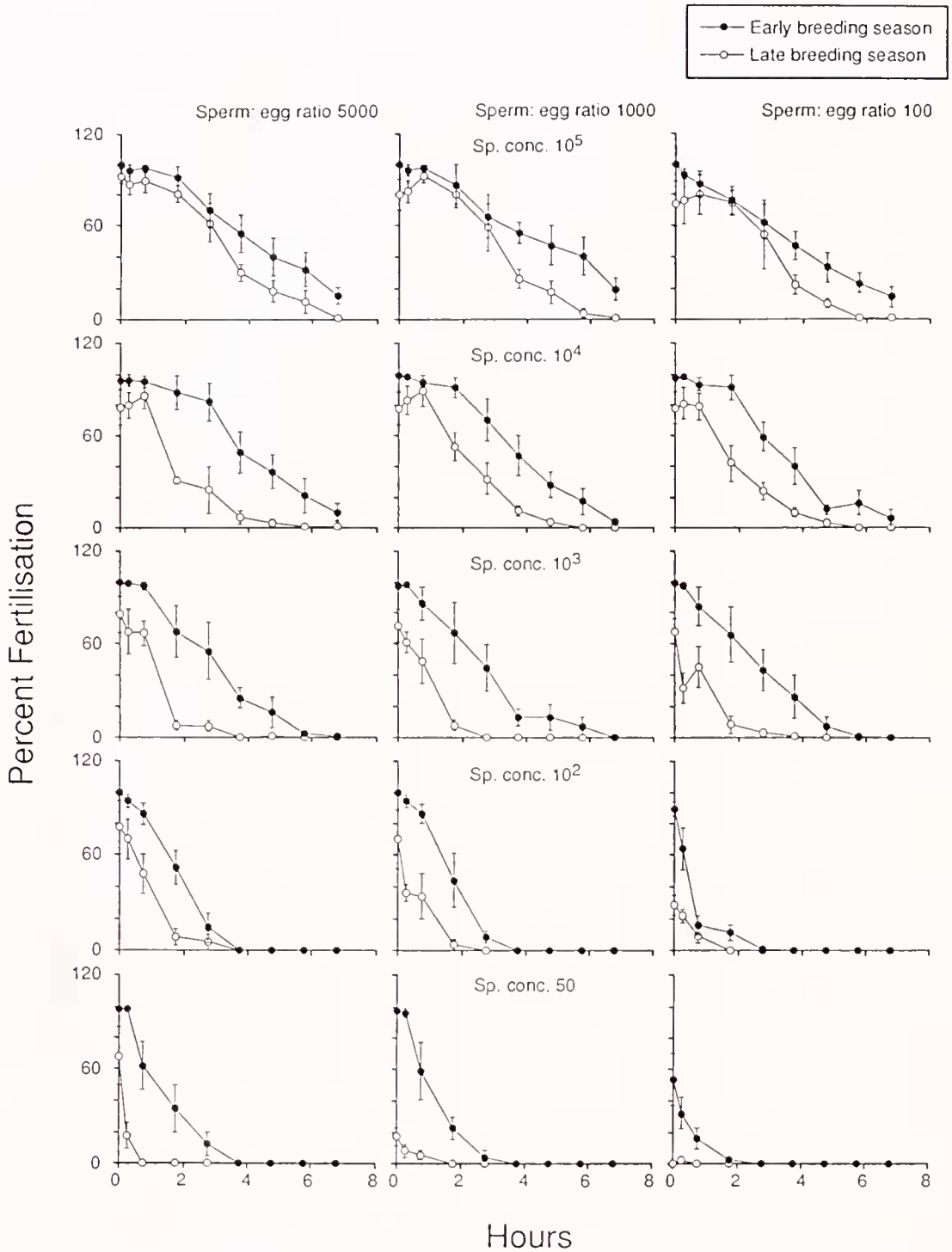

Figure 1. Mean percent fertilization, with standard error, of eggs from *Acanthaster planci* when mixed with sperm at different sperm concentrations (rows) and sperm:egg ratios (columns) over a period of 6 h 45 min. Sperm concentration varied from 50 to 10^5 sperm ml^{-1}, and sperm:egg ratio varied from 100 to 5000. Data from matings early (November–December) and late (January) in the breeding season are plotted separately.

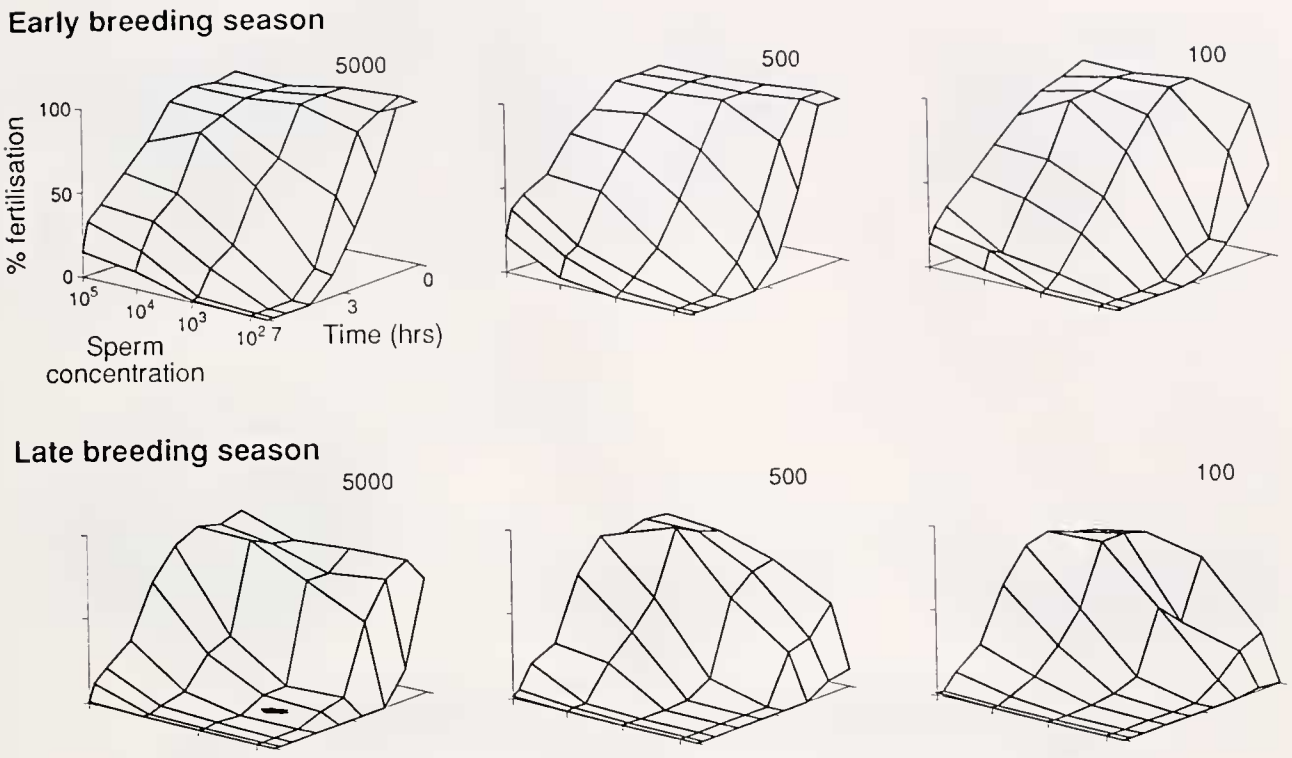

Figure 2. Fertilization response surfaces over a series of sperm concentrations and times since gamete release, plotted for three different sperm:egg ratios for the early and late breeding season.

times from gamete release. The overall geometry of the surface for the sperm:egg ratio of 500 from the late breeding season was depressed relative even to the sperm:egg ratio of 100 from the early breeding season, although its general form was similar. The two-dimensional surface of fertilization in these illustrations appears to be the result of the orthogonal interaction of two sets of sigmoid curves.

The temporal dynamics of fertilization have been summarized in a series of fertilization rate response surfaces plotted at different times from gamete release as a function of sperm:egg ratio and sperm concentration (Fig. 3). Data for the early breeding season show a plateau of high fertilization in the first 45 min, with a drop in fertilization observed only for the lowest sperm:egg ratios and sperm concentrations. The general decline in fertilization with time was more rapid for low sperm concentrations and varied little with sperm:egg ratio. When data from early and late breeding season were compared, the consistently lower fertilization rates and the more rapid decline in fertilization rate with time and at lower sperm concentrations in the late breeding season were clear (Fig. 4). The response surface for the late breeding season also tilted downwards more strongly at the apex of the sperm:egg ratio and sperm concentration axes than that from the early breeding season and folded downwards at relatively higher sperm:egg ratios (compare the 15-min and 45-min graphs in Figs. 3 and 4).

Gamete aging experiments

The gamete aging experiments included data only from the early breeding season and therefore did not require inclusion of time in breeding season as a factor in the analysis. Time since the aging gametes were first released (time), egg type (fresh or aged), sperm type (fresh or aged), sperm:egg ratio, and sperm concentration all had highly significant main effects (Table IV). Fertilization rates decreased with time since gamete release, with aged as opposed to fresh eggs or sperm, with decreasing sperm:egg ratio, and with decreasing sperm concentration (Fig. 5). There were highly significant two-way interactions of sperm concentration with all other factors, reflecting the greater reduction in fertilization rate at lower sperm concentrations with increasing time from gamete release, aged relative to fresh gametes, and decreasing sperm:egg ratio. Fertilization rates were also increasingly depressed the older the aged eggs or aged sperm used, leading to the significant two-way interactions between time since gamete release, egg type, and sperm type.

When the treatments using different sperm concentrations were analyzed separately, both showed significant

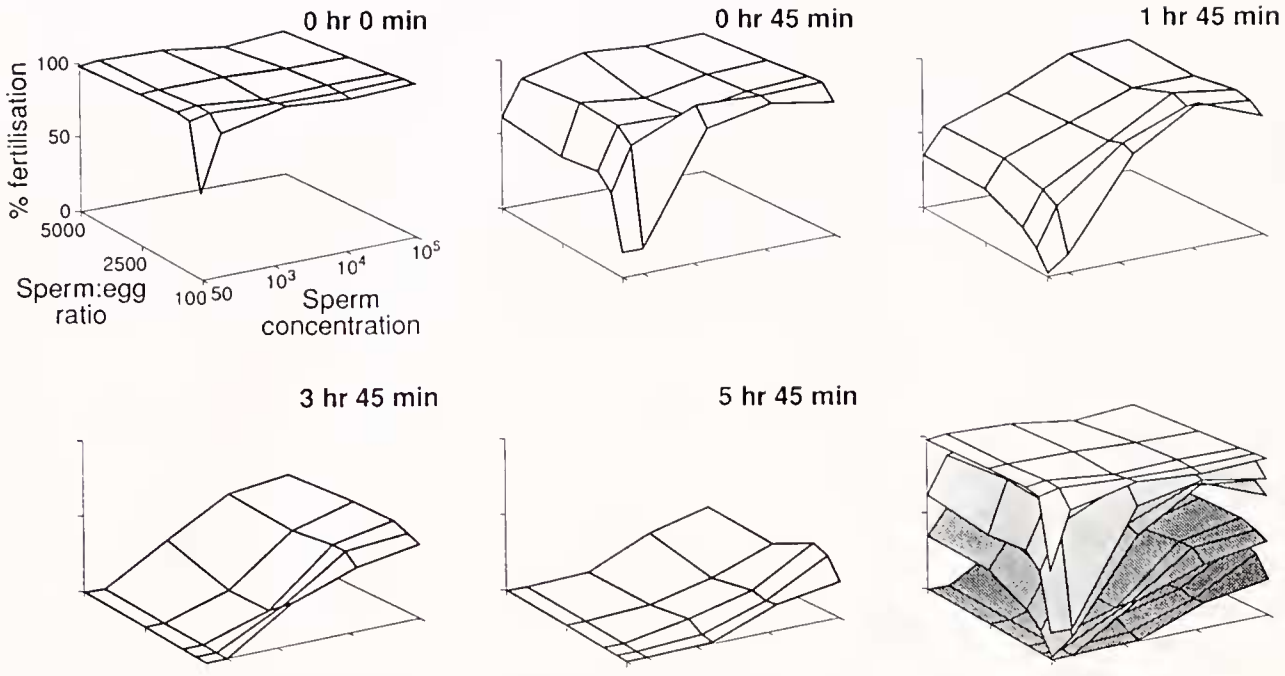

Figure 3. Fertilization response surfaces over a series of sperm concentrations and sperm:egg ratios, plotted at different times from gamete release. The set of six graphs depict the decline in fertilization early in the breeding season, with the sixth graph providing a key to the relative positions of the surfaces shown in the preceding five graphs.

main effects of time since gamete release, egg type, sperm type, sperm:egg ratio, and sperm concentration; both also showed the same suite of significant two-way interactions. However, the two-way interaction of egg type and sperm:egg ratio was nonsignificant in the 10^2 sperm ml^{-1} analysis, and that of sperm type and sperm:egg ratio was nonsignificant in the 10^4 sperm ml^{-1} analysis (Table IV). Far fewer higher order interactions were significant in each case.

The results clearly demonstrated an aging of both gametes, with aging eggs fertilized using fresh sperm showing a decline in fertilization rate after 3 h, dropping to 20% by 7 h (Fig. 5). The aging rate of sperm was much faster and dependent upon sperm concentration. At sperm concentrations of 10^2 sperm ml^{-1}, the fertilization rates of fresh eggs fertilized by aging sperm declined after 1 h, dropping to 0% after 3 to 4 h. At sperm concentrations of 10^4 sperm ml^{-1}, fresh eggs fertilized by aging sperm showed a decline in fertilization rate after 3 h, dropping to 20% by 7 h. Thus at 10^4 sperm ml^{-1}, aging eggs fertilized by aging sperm showed a fertilization rate similar to that of aging eggs and fresh sperm. At high sperm concentrations, fertilization rates were therefore largely determined by egg aging. In contrast, at 10^2 sperm ml^{-1}, aging eggs fertilized by aging sperm showed a pattern similar to that of fresh eggs fertilized by aging sperm. At low sperm concentrations, fertilization rates were therefore determined to a large extent by sperm aging.

Discussion

In this investigation, the first detailed analysis of fertilization dynamics of a starfish, *A. planci* showed general similarities to sea urchins—but there were also marked differences. The relative effects of sperm concentration, gamete age, and sperm:egg ratios on fertilization success in *A. planci* were similar to those reported for several species of sea urchin. Egg density or sperm:egg ratio had little influence on fertilization success, but gamete age and sperm concentration had marked effects, as in sea urchins (Lillie, 1915; Dinnel *et al.*, 1987; Levitan *et al.*, 1991). However, fertilization dynamics in *A. planci* appeared to be more sensitive to sperm:egg ratio than are the sea urchins studied to date. Although Levitan *et al.* (1991) found no significant effect of egg concentration over the range they examined, they used a model developed by Vogel *et al.* (1982) to predict reduced fertilization success for *Strongylocentrotus franciscanus* at low sperm concentrations of 10^2 sperm ml^{-1} (10 sperm μl^{-1}) and high egg concentrations of 10^4 eggs ml^{-1} (10^3 eggs μl^{-1}), equaling sperm:egg ratios of 0.01 or less (see Fig. 8 of Levitan *et al.*, 1991). Effects on fertilization success of *A. planci* were observed at far higher sperm:egg ratios (up to 100 early in the breeding season and up to 500 late in the breeding season) for sperm concentrations of 10^2 sperm ml^{-1} (Fig. 3).

The fertilization response surfaces for the sea urchin *S. franciscanus* (Levitan *et al.*, 1991) and the starfish *A. planci*

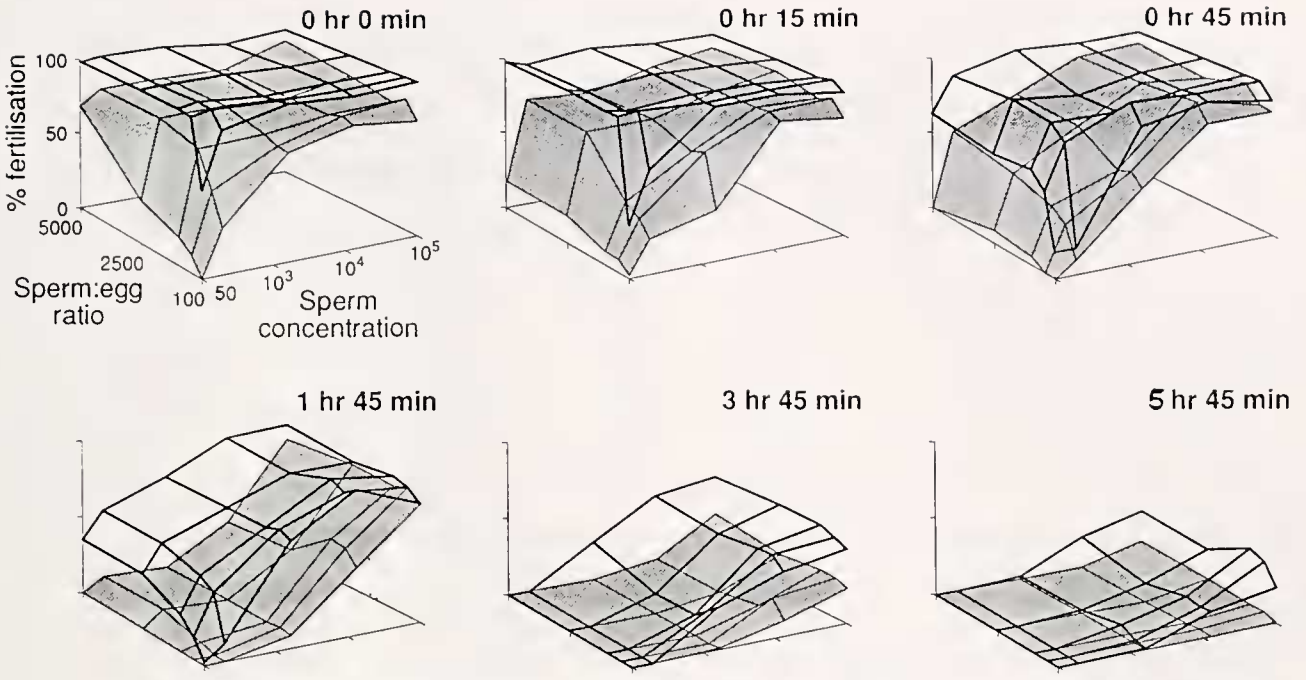

Figure 4. Fertilization response surfaces over a series of sperm concentrations and sperm:egg ratios, plotted at different times from gamete release and comparing the fertilization response surface early (transparent surface) and late (stippled surface) in the breeding season.

were similar in that fertilization success declined with declining sperm concentrations and increasing time from gamete release. The loss of fertilizing capacity of sperm with dilution is called the respiratory dilution effect (Chia and Bickell, 1983) and results from the fact that although the total amount of oxygen consumed by a spermatozoan over its lifespan is independent of dilution, the rate of consumption is far greater at low sperm concentrations, leading to a shorter lifespan at low concentrations. A dilution effect was clearly demonstrated for *A. planci*.

However, the dynamics of fertilization of *A. planci* were very different from those of *S. franciscanus*. Fertilizing capacity was lost more rapidly by *S. franciscanus* at given sperm concentrations and declined more rapidly with increasing dilution of sperm. At sperm concentrations of 10^5 sperm ml^{-1}, fertilization rates for *A. planci* early in the breeding seasons were above 90% for up to 105 min from the time of gamete release, but rates for *S. franciscanus* had declined from over 90% at 10 min to approximately 40% by 80 min (compare Fig. 1 from the present paper with Fig. 2 of Levitan *et al.*, 1991). Similarly, at sperm concentrations of 10^3 sperm ml^{-1}, fertilization rates for *A. planci* were above 90% for up to 45 min, declining to 50% by 105 min; but rates for *S. franciscanus* were less than 40% at time zero, declining to less than 10% by 80 min. Fertilization success in *A. planci* at time zero showed no decline at sperm concentrations from 10^5 to 10^3 sperm ml^{-1}, but fertilization in *S. franciscanus* fell from almost 100% to approximately 30% over the same range. The fertilization dynamics inferred from more restricted data reported for a range of sea urchin species (Lillie, 1915; Gray, 1928; Pennington, 1985) were comparable to that reported for *S. franciscanus* by Levitan *et al.* (1991). Fertilization dynamics cannot be further compared using parameters, such as β, from the Vogel *et al.* (1982) model because these parameters require data on the fertilization success achieved at different gamete contact times, and these data are not available for *A. planci*.

A. planci sperm aged more rapidly than eggs, at least at concentrations likely to be encountered in the field; similar results have been described for the sea urchin *S. droebachiensis* (Pennington, 1985). Detailed data on the independent aging of sea urchin sperm and eggs are lacking, making it impossible to establish whether the extent to which egg aging determines fertilization dynamics at high sperm concentrations and sperm aging determines fertilization dynamics at low sperm concentrations is similar in sea urchins and starfish. It is clear that eggs of the crown-of-thorns starfish are capable of being fertilized for up to several hours after they are first released. *A. planci* sperm also retain their fertilizing capacity for 1 to 2 h even at reasonably dilute concentrations: this is because the sperm of *A. planci* are less affected by the reduced respiratory dilution effect than are sea urchin sperm.

A greater sensitivity of fertilization to sperm:egg ratio and a relatively slow rate of gamete aging appear to char-

Table IV

Analysis of variance testing the effects of time since initial gamete release (time), egg type (whether fresh or aged), sperm type (whether fresh or aged), sperm:egg ratio, and sperm concentration, on fertilization success of Acanthaster planci

	Total data set			Sperm concentration = 10^2			Sperm concentration = 10^4		
Source of variance	Degrees of freedom (DF)	Mean square (MS)	$F_{0.07}$	DF	MS	$F_{0.08}$	DF	MS	$F_{0.06}$
Time (T)	6	11.17	161.1***	6	5.08	60.7***	6	6.93	126.2***
Egg type (E)	1	25.71	370.9***	1	4.83	57.8***	1	24.71	449.9***
Sperm type (S)	1	45.12	650.9***	1	66.51	794.6***	1	1.81	32.9***
Sperm:egg ratio (R)	2	8.63	124.5***	2	10.24	122.4***	2	0.91	16.8***
Sperm concentration (C)	1	44.95	648.4***	–	–	–	–	–	–
T × E	6	2.38	34.4***	6	0.50	5.9***	6	2.33	42.5***
T × S	6	2.04	29.4***	6	2.60	31.3***	6	0.38	6.8***
E × S	1	2.24	32.3***	1	2.50	30.1***	1	0.28	5.1***
T × R	12	0.22	3.1***	12	0.61	7.3***	12	0.18	3.2***
E × R	2	0.39	5.6***	2	0.03	0.4^{NS}	2	0.66	12.1***
S × R	2	0.55	7.8***	2	0.87	10.4***	2	0.14	2.5^{NS}
T × C	6	0.85	12.3***	–	–	–	–	–	–
E × C	1	3.84	55.4***	–	–	–	–	–	–
S × C	1	23.20	334.7***	–	–	–	–	–	–
R × C	2	2.53	36.5***	–	–	–	–	–	–
T × E × S	6	0.59	8.6***	6	0.54	6.5***	6	0.17	3.2**
T × E × C	6	0.45	6.5***	–	–	–	–	–	–
T × E × R	12	0.06	0.8^{NS}	12	0.06	0.7^{NS}	12	0.06	1.1^{NS}
T × S × R	12	0.23	3.3***	12	0.51	6.1***	12	0.05	1.0^{NS}
T × S × C	6	0.93	13.5***	–	–	–	–	–	–
E × S × R	2	0.15	2.1^{NS}	2	0.14	1.7^{NS}	2	0.06	1.1^{NS}
E × S × C	1	0.56	8.1**	–	–	–	–	–	–
T × R × C	12	0.57	8.2***	–	–	–	–	–	–
E × R × C	2	0.31	4.4*	–	–	–	–	–	–
S × R × C	2	0.45	6.5**	–	–	–	–	–	–
T × E × S × R	12	0.04	0.6^{NS}	12	0.11	1.3^{NS}	12	0.03	0.5^{NS}
T × E × S × C	6	0.13	1.8^{NS}	–	–	–	–	–	–
T × E × R × C	12	0.07	0.9^{NS}	–	–	–	–	–	–
T × S × R × C	12	0.34	4.9***	–	–	–	–	–	–
E × S × R × C	2	0.06	0.8^{NS}	–	–	–	–	–	–
T × E × S × R × C	12	0.09	1.3^{NS}	–	–	–	–	–	–
Error	792	0.07		396	0.08		396	0.06	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{NS} not significant.

acterize *A. planci* gametes. However, the extent to which these factors influence fertilization success in the wild depends upon the range of values encountered in the field and their interaction with other variables. For example, Levitan *et al.* (1991) concluded that egg concentration was unlikely to be an important determinant of fertilization success in the sea urchin *S. franciscanus*, except within the first few seconds of gamete release, because of the concentration at which eggs were extruded and the rapidity with which the eggs were diluted by seawater. Assuming that *A. planci* eggs are extruded in a column from each gonopore to give a concentration of 2500 ml^{-1}, the packing density for eggs of 200-μm diameter is at a maximum, and sperm are extruded at a concentration of 500×10^6 sperm ml^{-1}, the sperm:egg ratio would be 200,000—well above the value at which sperm:egg ratio affects fertilization success. Thus, as in *S. franciscanus*, sperm:egg ratio in *A. planci* is unlikely to play a major role in determining fertilization success in the field.

Similarly, the dilution at which sperm are unable to fertilize eggs is likely to be reached well before eggs lose the ability to be fertilized. The dilution of sperm has been shown to be rapid within only a few meters (Denny, 1988; Denny and Shibata, 1989) and to have a major influence on observed fertilization success in a number of marine invertebrates (Pennington, 1985; Yund, 1990; Grosberg, 1991; Levitan, 1991; Levitan *et al.*, 1992). Indeed, even in slow currents, such dilution is likely to occur well within the lifespan of *A. planci* sperm at concentrations as low as 50 sperm ml^{-1}. Gamete lifespan is therefore unlikely to be a major factor in limiting fertilization success over

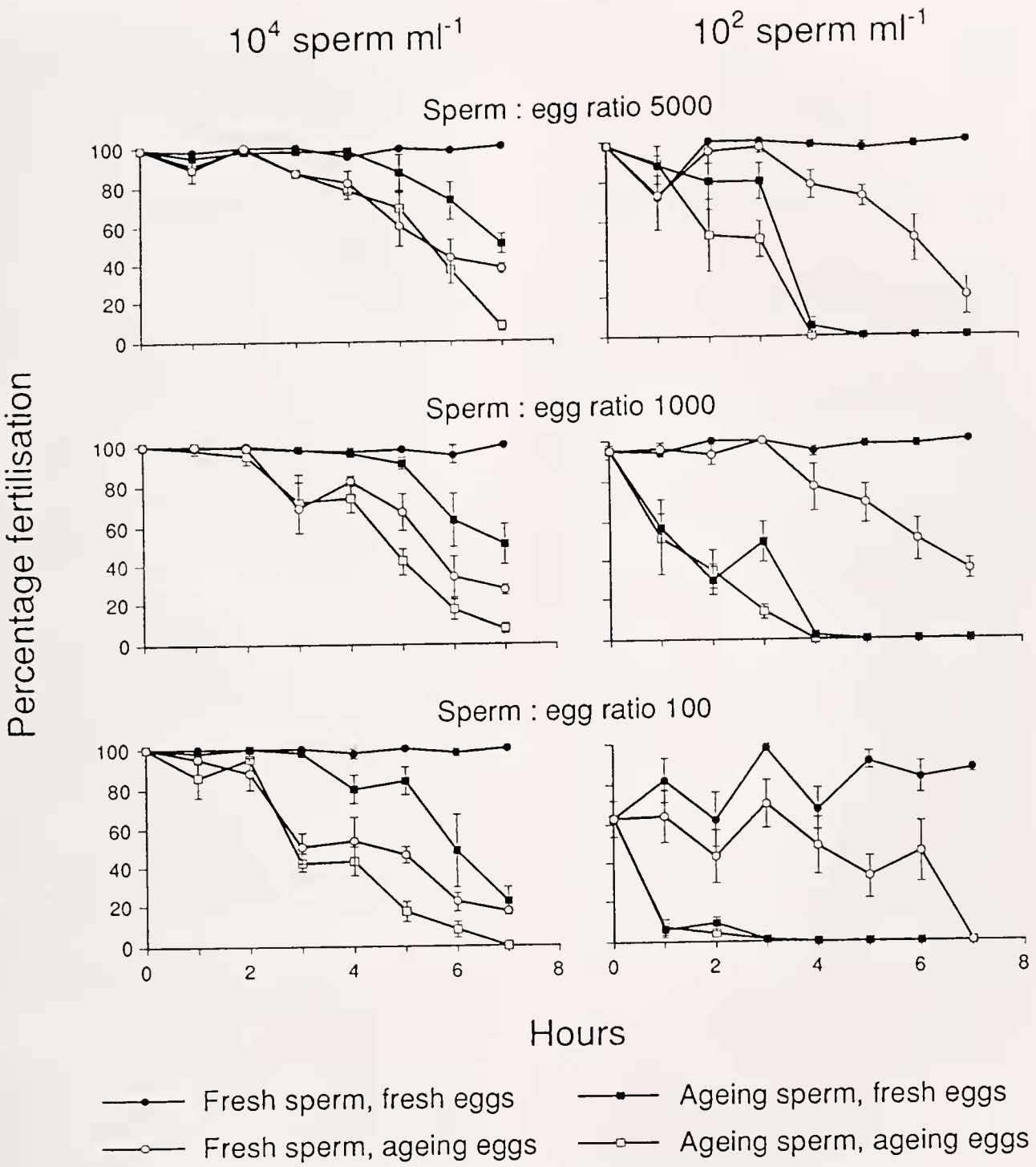

Figure 5. Mean percent fertilization, with standard error, of fresh eggs and aged eggs from *Acanthaster planci* when mixed with fresh and aged sperm at different sperm concentrations (rows) and sperm:egg ratios (columns) over a period of 6 h and 45 min. Sperm concentration was either 10^2 or 10^4 sperm ml^{-1}, and sperm:egg ratio was either 100, 1000, or 5000.

small spatial scales, but the respiratory dilution effect may be critical.

Detailed biochemical work with *Strongylocentrotus purpuratus* has demonstrated that various treatments used to activate sperm all give rise to an increased intracellular pH (Christen *et al.*, 1983a, 1986; Johnson *et al.*, 1983). Sea urchin sperm can be maintained for a long time in a quiescent, "non-aging" state at the pH of seminal fluid, that is, an extracellular pH of approximately 7.5 and an intracellular pH of approximately 7.0 (Christen *et al.*, 1983b). These findings explain the effect of a number of artificial means of inducing sperm motility as well as the extended fertilizing capacity observed for sperm in buffered solutions (Timourian and Watchmaker, 1970; Christen *et al.*, 1983a; Johnson *et al.*, 1983). Sperm are activated in nature when an intracellular pH higher than their activation threshold (approximately pH 7.5) develops after they are released into seawater where the external pH is 8.0 or more. Christen *et al.* (1983a, 1986) have demonstrated that the effects of short exposure to circumstances that activate the sperm are reversible as long as high energy compounds used in respiration are not sufficiently depleted to damage cell function. High pH activates both microtubule movement that depletes ATP and mitochondrial reactions that produce ATP. The former (consumption) is usually more rapid than the latter (production), and the relative rate of these processes determines the time for which sperm are viable at a given environmental pH, and whether the situation is reversible.

Variations in external pH may therefore influence the fertilization success in experiments. However, no major variations in seawater pH were observed in our experiments, and in any case we wished to determine how varying gamete age and concentration would affect fertilization in environments likely to occur in the wild. The biochemical literature is pertinent to the work we report in that it helps explain the mechanisms of sperm aging and allows us to speculate on specific reasons for the difference in sperm aging observed in *A. planci*.

We did not measure the pH of the sperm solution immediately after spawning (when sperm concentrations were $12–40 \times 10^8$ sperm ml^{-1}) or in the stock solution (with 2×10^6 sperm ml^{-1}). However, our observations are consistent with the view that any aging effects of dilution of sperm during the early stages of spawning were reversed when sperm concentrations increased (and possibly external pH decreased) with continuing addition of sperm to the 100 ml of seawater in the beaker. When sperm were released, they accumulated in a thick sludge at the base of the beaker and were therefore very concentrated. The values of $12–40 \times 10^6$ sperm ml^{-1} were observed after the seawater in the beaker was mixed very well at the end of the spawning period. Our observations are also consistent with the view that respiration was at least balanced by ATP production at 2×10^6 sperm ml^{-1}. There was no correlation between initial concentrations at which sperm had been spawned and the relative speed of aging of the sperm in subsequent experiments that might suggest the initial spawning into seawater had irreversibly aged sperm. The fact that *A. planci* sperm held at concentrations of 2×10^6 sperm ml^{-1} do not appear to age over several hours, whatever the reason, is in itself evidence that their fertilization capacity is enhanced relative to that of sea urchin sperm, whose viability extended over several hours only at concentrations of 10^8 sperm ml^{-1} (Levitan *et al.*, 1991).

Our conclusions depend on the use of "non-aged" sperm at the start of our experiments, and our empirical protocols allowed us to do this. The experiments were carried out with natural seawater pumped fresh from the Great Barrier Reef lagoon, replicating the conditions in which natural fertilization would occur and allowing us to estimate fertilization rates in the wild between gametes of given age and concentration. The results do not allow us to identify the mechanisms that give rise to the different rates of aging of sea urchin and crown-of-thorns starfish sperm.

However, one may speculate that, compared to sea urchins, the crown-of-thorns starfish might have (1) more acid seminal fluid, resulting in lower seawater pH at given sperm concentrations; (2) higher pH thresholds for activation of sperm; (3) different rates of reaction for respiration and ATP production with rise in pH, resulting in longer viability; or (4) combinations of the above.

The conclusions are also conservative in that gamete age was calculated from the time of dilution from the stock solution. This was shown to be an appropriate empirical starting point because *A. planci* sperm do not age at this concentration over the time scales of the experiment. If age had been timed from the beginning of sperm release, the sperm would have been considered some 30 min older on average, thus increasing the observed difference in fertilization rates of *A. planci* and sea urchin sperm.

In the field, fertilization rates as high as about 60% at 10 m downstream of a spawning male, dropping to 20% at 60 m distance, have been observed for *A. planci* (Babcock and Mundy, 1992). These values are much greater than those of sea urchins, which show 5% fertilization at 5 m, dropping to zero at distances greater than 10 m (Pennington, 1985). The reduced respiratory dilution effect, and consequent relatively slow aging of sperm, are features of *A. planci* that may well enhance fertilization success by allowing gametes to remain competent for a longer time at more dilute concentrations. Environmental factors such as salinity, temperature, and pH are also likely to affect fertilization success (Rupp, 1973; Greenwood and Bennett, 1981; Dinnel *et*

al., 1987; Christen *et al.*, 1983a, 1986), and hydrodynamics and rates of gamete release will control the degree of dilution of the gametes. However, the characteristics of *A. planci* gametes described here are very different from those of sea urchins and would lead to higher fertilization success at greater distances from spawning adults. The range of gamete concentrations tested was similar to the average values of 2×10^5 sperm ml^{-1} or less, and sperm:egg ratios of 2×10^4 or less, measured in the field (Benzie *et al.*, 1994). The nature of gamete dispersion in the wild and estimates of fertilization success in the wild, using field measurements of gamete concentrations and the laboratory data presented here, are fully discussed in Benzie *et al.* (1994).

A. planci larvae developed from fertilization at the highest laboratory concentration of 10^5 sperm ml^{-1} were often malformed and had not completed gastrulation the following day, in contrast to larvae from treatments using lower sperm concentrations. No extensive rearing of larvae was attempted, but these observations from several tests suggest that polyspermy might have occurred at high sperm concentrations. Individuals of *A. planci* in close proximity in the field are likely to produce concentrations of gametes that will result in some degree of polyspermy. The high fertilization rates achieved may not reflect the number of viable larvae produced in such circumstances, and there may be an upper limit to sperm concentrations with respect to the number of viable larvae produced in a given mating. Similarly, although eggs were fertilized up to 8 h after their release, after only 4 to 5 h many showed abnormal divisions, and eggs were often misshapen. When samples were maintained for 36 h, many of the eggs with raised fertilization membranes had not developed into larvae or had not successfully completed gastrulation.

The viability of eggs fertilized at the end of the breeding season must also be assessed. Fertilization dynamics early and late in the breeding season have never been comprehensively compared for any echinoderm species. Differences observed in the fertilization rate response surfaces early and late in the breeding season suggested that a reduction in the quality of gametes at the end of the breeding season gave rise to different fertilization dynamics in *A. planci* at that time. It is not known whether eggs, sperm, or both were responsible for these differences.

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Literature Cited

Babcock, R., and C. Mundy. 1992. Reproductive biology, spawning and field fertilization rates of *Acanthaster planci*. *Aust. J. Mar. Freshwater Res.* **43:** 525–534.

Benzie, J. A. H., K. P. Black, P. J. Moran, and P. Dixon. 1994. Small-scale dispersion of eggs and sperm of the crown-of-thorns starfish (*Acanthaster planci*) in a shallow coral reef habitat. *Biol. Bull.* **186:** 153–167.

Chia, F.-S., and L. R. Bickell. 1983. Echinodermata. Pp. 545–620 in *Reproductive Biology of Invertebrates, Vol II: Spermatogenesis and Sperm Function*, K. G. Adiyondi and R. G. Adiyondi, eds. John Wiley and Sons, New York.

Christen, R., R. W. Schackmann, and B. M. Shapiro. 1983a. Metabolism of sea urchin sperm, interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. *J. Biol. Chem.* **258:** 5392–5399.

Christen, R., R. W. Schackmann, F. W. Dahlquist, and B. M. Shapiro. 1983b. ^{31}P-NMR analysis of sea urchin sperm activation, reversible formation of high energy phosphate compounds by changes in intracellular pH. *Exp. Cell. Res.* **149:** 289–294.

Christen, R., R. W. Schackmann, and B. M. Shapiro. 1986. Ionic regulation of sea urchin sperm motility, metabolism and fertilizing capacity. *J. Physiol.* **379:** 347–365.

Cohn, E. 1918. Studies on the physiology of spermatozoa. *Biol. Bull.* **34:** 167–218.

Denny, M. W. 1988. *Biology and the Mechanics of the Wave-swept Environment.* Princeton University Press, New Jersey, 329 pp.

Denny, M. W., and M. F. Shibata. 1989. Consequences of surf zone turbulence for settlement and external fertilization. *Am. Nat.* **134:** 859–889.

Dinnel, P. A., J. M. Link, and Q. J. Stober. 1987. Improved methodology for a sea urchin sperm cell bioassay for marine waters. *Arch. Environ. Contam. Toxicol.* **16:** 23–32.

Dixon, W. J. (ed.). 1983. *BMDP Statistical Software.* University of California Press, Berkeley, 734 pp.

Gray, J. 1928. The effect of dilution on the activity of spermatozoa. *J. Exp. Biol.* **5:** 337–344.

Greenwood, P. J., and T. Bennett. 1981. Some effects of temperature-salinity combinations on the early development of the sea urchin *Parechinus angulosus* (Leske) fertilization. *J. Exp. Mar. Biol. Ecol.* **51:** 119–131.

Grosberg, R. K. 1991. Sperm-mediated gene flow and the genetic structure of a population of a colonial ascidian *Botryllus schlosseri*. *Evolution* **45:** 130–142.

Ishikawa, M. 1975. Fertilization. Pp. 99–147 in *The Sea Urchin Embryo: Biochemistry and Morphogenesis*, G. Czihak, ed. Springer-Verlag, Heidelberg.

Johnson, C. H., D. L. Clapper, M. W. Winkler, H. C. Lee, and D. Epel. 1983. A volatile inhibitor immobilizes sea urchin sperm in semen by depressing intracellular pH. *Develop. Biol.* **98:** 493–501.

Kanatani, H., and Y. Nagahama. 1983. Echinodermata. Pp. 611–654 in *Reproductive Biology of Invertebrates, Vol I: Oogenesis, Oviposition and Oosorption*, K. G. Adiyondi and R. G. Adiyondi, eds. John Wiley and Sons, New York.

Kobayashi, N. 1984. Marine ecotoxicological testing with echinoderms. Pp. 341–405 in *Ecotoxicological Testing for the Marine Environment, Vol. I*, G. Persoone, E. Jaspers, and C. Claus, eds. State University of Ghent and Institute of Marine Scientific Research, Bredene, Belgium.

Lassig, B. R., and G. Kelleher. 1991. Crown-of-thorns starfish on the Great Barrier Reef. Pp. 39–62 in *Environmental Research in Aus-*

tralia. Case Studies. Australian Science and Technology Council. Australian Government Publishing Service. Canberra.

Levitan, D. R. 1991. Influence of body size and population density on fertilization success and reproductive output in a free-spawning invertebrate. *Biol. Bull.* **181:** 261–268.

Levitan, D. R., M. A. Sewell, and F.-S. Chia. 1991. Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus:* interaction of gamete dilution, age and contact time. *Biol. Bull.* **181:** 371–378.

Levitan, D. R., M. A. Sewell, and F.-S. Chia. 1992. How distribution and abundance influence fertilization success in the sea urchin *Strongylocentrotus franciscanus. Ecology* **73:** 248–254.

Lillie, F. R. 1915. Studies of fertilization. VII. Analysis of variations in the fertilizing power of sperm suspensions of *Arbacia. Biol. Bull* **28:** 229–251.

Lillie, F. R. 1919. *Problems of Fertilization* University of Chicago Press, Chicago. 278 pp.

Lucas, J. S. 1982. Quantitative studies on the feeding and nutrition during larval development of the coral reef asteroid *Acanthaster planci* (L.). *J. Exp. Mar. Biol. Ecol* **65:** 173–193.

Meijer, L., and P. Guerrier. 1984. Maturation and fertilization in starfish oocytes. *Int. Rev. Cytology* **86:** 129–196.

Moran, P. J. 1986. The *Acanthaster* phenomenon. *Oceanogr. Mar. Biol. A. Rev* **24:** 379–480.

Pennington, J. T. 1985. The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning. *Biol. Bull.* **169:** 417–430.

Rupp, J. H. 1973. Effects of temperature on fertilization and early cleavage of some tropical echinoderms, with emphasis on *Echinometra mathaei. Mar. Biol.* **23:** 183–189.

SAS Institute Inc. 1985. *SAS User's Guide: Statistics.* Version 5 edition. SAS Institute Inc., Cary, NC. 1028 pp.

Timourian, H., and G. Watchmaker. 1970. Determination of spermatozoan motility. *Develop. Biol.* **21:** 62–72.

Tyler, A., and B. S. Tyler. 1966. The gametes: some procedures and properties. Pp. 639–682 in *Physiology of Echinodermata.* R. A. Boolootian, ed., Interscience Publishers, New York.

Vogel, H., G. Czihak, P. Chang, and W. Wolf. 1982. Fertilization kinetics of sea urchin eggs. *Math. Biosci.* **58:** 189–216.

Yamaguchi, M. 1974. Growth of juvenile *Acanthaster planci* (L.) in the laboratory. *Pac. Sci.* **28:** 123–138.

Yund, P. O. 1990. An *in situ* measurement of sperm dispersal in a colonial marine hydroid. *J. Exp. Zool.* **253:** 102–106.